# Application Note

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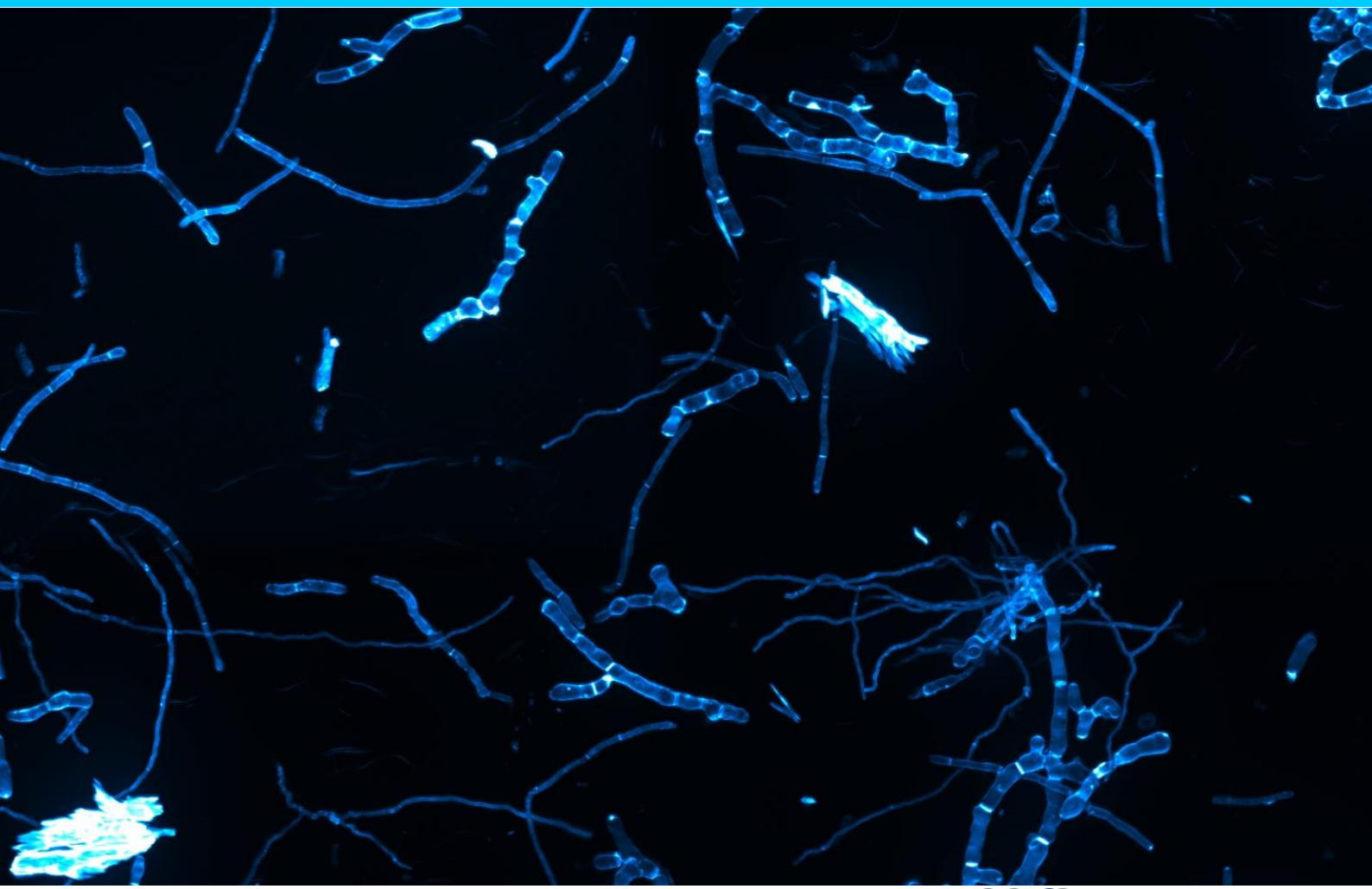
# High throughput cultivation of the cellulolytic fungus *Trichoderma reesei* in the BioLector®



Leibniz Institute
for Natural Product Research and Infection Biology
Hans Knoell Institute

## Cultivation of filamentous fungi

Filamentous fungi play an important role in the industrial biotechnology. They are attractive candidates for the production of organic acids, enzymes as well as secondary metabolites. *Aspergillus niger* for example is used for the citric acid production and for the production of amylases, catalases and proteases. In general, 38 % of all known bioactive metabolites originate from filamentous fungi [1,2]. One of the most important industrial producers for cellulases and hemicellulases is *Trichoderma reesei* (*T. reesei*). It has a high protein secreting capacity for cellulases up to 100 g per liter while it grows on inexpensive lignocellulosic materials [3,4]. Furthermore, it is used as model organism for plant biomass degradation.

Classically, *T. reesei* is cultivated in shake flasks with only limited experimental throughput. High throughput cultivation of filamentous fungi in microtiter plates has been proven difficult due to their morphologic complexity. Depending on the hydromechanical stress and various other factors, mycelia can either grow freely dispersed or form macroscopic aggregates from mycelial clumps to pellets. Thereby, morphology has great impact on productivity, growth kinetics and physical properties of the fermentation broth. Dispersed mycelium enables a homogenous supply of nutrients to the culture but tremendously increases the viscosity of the broth, impeding efficient aeration. In contrast, pellets cause a lower viscosity but lead to inhomogeneous supply of nutrients with well supplied pellet periphery and starving pellet cores [5,6]. Consequently, suitable morphology has to be identified for each strain and process individually [7]. Since the hydromechanical stress is typically low in small scale systems compared to shake flasks and large stirred tank fermenters, the morphology in these different cultivation systems can often differ significantly. For scale-down of filamentous cultures, it is therefore important to adjust the cultivation parameters not only based on classical bioprocess scaling variables like the gas transfer coefficient kLa or volumetric power input, but also based on morphology. For microtiter plates this can be mainly achieved by variation of the shaking speed, well diameter and introduction of baffles (e.g. flower-plate design). For extremely viscous cultures it is further necessary to increase the shaking diameter to prevent out of phase phenomena that will impede aeration. For cultures that are preferentially grown as dispersed mycelium like *T. reesei* but are prone to cell damage introduced by baffle, the 48-RoundWellPlate in combination with a high shaking speed offers a sufficiently large well diameter that causes enough shear stress to prevent clump formation while maintaining a high throughput.

In this application note we show, that the BioLector® system is suitable for high throughput fermentation of *T. reesei* while maintaining essentially the same morphology, cellulase production and growth kinetics as in conventional shake flask fermentations. Furthermore, we demonstrate different application examples that take advantage of the optical online measurement capabilities of the BioLector®. These examples include a reporter-based estimation of cellulase induction and online measurement of growth rates. This application note was realized in cooperation with M. Rosenbaum and I. Schlembach from the BioPilot Plant of the Leibniz Institute for Natural Product Research and Infection Biology - Hans Knoell Institute (HKI Jena, Germany).

## Methods

**Cultivation of *T. reesei* in the BioLector®:** The cultivation of *T. reesei* RUT-C30 RFP1 takes place in the BioLector® at 30° C cultivation temperature, 1200 rounds per minutes (rpm) shaking speed and 1000 µL filling volume per well in each cultivation well of the RoundWellPlate (type: MTP-R48-B).

**Cultivation of *T. reesei* in shake flasks:** The cultivation of *T. reesei* RUT-C30 RFP1 takes place in shake flasks at 30° C cultivation temperature, 300 rpm shaking speed at 50 mm shaking diameter and 20 mL filling volume per 250 mL flask.

**Cultivation medium:** For the cultivation medium, a modified mineral medium based on the medium published by Pakula et al. is used. [8]

The final medium exclusive carbon source consists of $(NH_4)_2SO_4$ 7.6 g/L, $KH_2PO_4$ 2.6 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, $CaCl_2 \cdot 2H_2O$ 0.23 g/L, NaCl 0.05 g/L, 1,4-Piperazinedipropanesulfonic acid (PIPPS) 33 g/L (0.1 M), urea 0.3 g/L, peptone ex casein 2 g/L (N-Z-Amine® AS, Tween 80 0.1% (v/v), trace element solution 2.5 mL/L.

For Application example C, $(NH_4)_2SO_4$, $KH_2PO_4$ and peptone concentration were varied and the medium was buffered to pH 6.7 with 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) instead of PIPPS buffer.

The trace element solution (400× concentrated) had the following composition: citric acid 180 g/L, $Fe_2(SO_4)_3$ 2.29 g/L, $ZnSO_4 \cdot 7H_2O$ 16 g/L, $CuSO_4$ 2.05 g/L, $MnSO_4 \cdot 7H_2O$ 1.6 g/L, $H_3BO_3$ 0.8 g/L, $CoCl_2 \cdot 6H_2O$ 2.71 g/L.

The main solution without trace elements and carbon source was always prepared as a 2× concentrated stock solution that was set to pH 5.5 with 5 M NaOH. The solution was filtered through a 0.2 µm filter for sterilization. Before the experiment, the 2× concentrate was diluted to its original concentration by addition of sterile bidest water and other supplementing solutions such as trace elements, carbon sources or spore inoculum.

**Inoculum:** All cultures were inoculated from aqueous stock spore suspensions to a final concentration of $10^6$ spores/mL.

**Sampling procedure:** For sampling, the BioLector® cultivation was shortly interrupted and a whole well was harvested. Then, the measurement was quickly resumed.

**Construction of the *T. reesei* RUT-C30 strain RFP1:** The red fluorescent protein (RFP) tagged strain of *T. reesei* Rut-C30 (RFP1) was transformed with an expression cassette consisting of a shortened version of the *chb1* promotor, an engineered fast folding variant of *Discosoma* RFP and the *cbh2* terminator.

**Cellulase enzyme assay:** The cellulase activity in the culture supernatant was measured by the standard filter paper activity (FPA) assay according to the method of Ghose [9] adapted by Xiao [10]. The assay was performed in 60 µL reaction volume in 96-well conical bottom PCR plates.

**Biomass calibration:** For the calibration between scattered light and fungal dry weight, the dry weight of the culture was determined first. Fungal broth was filled into conical bottom glass tubes that had been weighed before on a microbalance in empty state. The broth was dried over night at 40°C in a vacuum oven. Then, the dry weight was determined by calculating the weight difference between empty and filled glass tube. To check the linearity between fungal dry weight and scattered light, the scattered light signal of a dilution series of the culture was measured in the BioLector® and plotted against the corresponding fungal dry weight.

**Morphology analysis**: The morphology was analyzed by spinning disc fluorescence microscopy using Calcofluor white (100 mg/L) to stain fungal cell walls. The culture broth was diluted 1:100 in water and applied to a microscopy chamber (ibidi

µ-slide angiogenesis). A z-stack was recorded over 72 µm depth above the bottom of the microscope chamber. Then, a maximum projection of the z-stack was calculated using the image calculator tool FIJI to extend the depth of field.

## Results

**Comparison of growth and cellulase production of *T. reesei* between BioLector® and shake flask cultivations**: Comparability between shake flask cultivation and BioLector® cultivation was verified by investigating cellulase production under three different conditions in both systems. Different degrees of cellulase induction were achieved using three different carbon sources: 30 g/L glucose for cellulase repressing conditions, 30 g/L lactose for slight cellulase induction and 30 g/L α-cellulose for full cellulase inducing conditions [11] Cellulase production and cultivation pH were then measured offline from samples harvested at different time points. As expected, depending on the used carbon source, different amounts of cellulase were produced (see figure1A). The cellulase production was closely matched in both systems, confirming the suitability of the BioLector® system for screening cellulase production. The pH profiles of the BioLector® cultivation closely matched the pH profiles of the shake flask cultivations (see figure1B) as well. As the pH drop is related to $NH_4^+$ consumption and, in turn, cell growth, this result indicates very comparable growth profiles of the fungus in both systems. Importantly, also the fungal morphology was comparable between BioLector® and shake flasks as shown in figure 2.

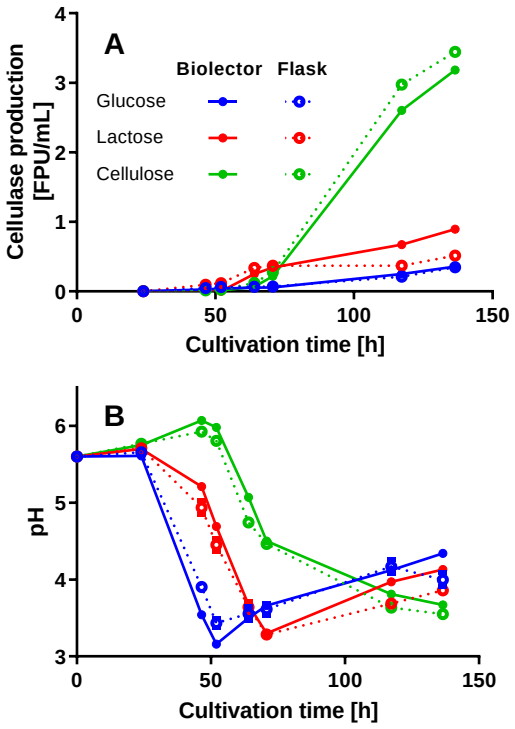



**Figure 1: Comparison BioLector® and traditional shake flask cultivations of *T. reesei* RUT-C30 RFP1 with different carbon sources.** (A) Comparison of cellulase production; (B) Comparison of pH profiles. The growth medium was supplemented with 30 g/L of the tested carbon sources. Data show mean values of 3 biological replicates for shake flask cultivations and single harvested wells for BioLector® cultivations.

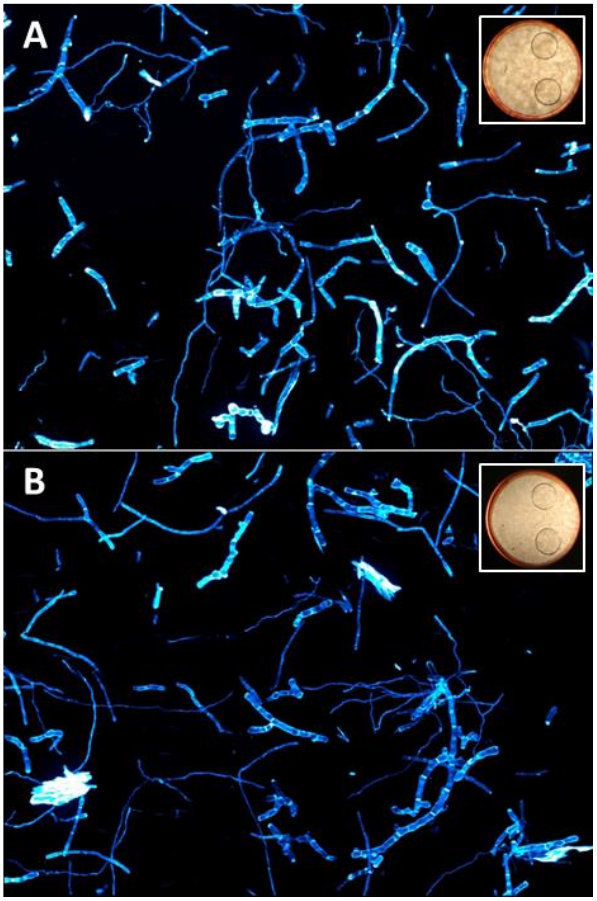



**Figure 2: Comparison of micro and macro morphology of *T. reesei* RUT-C30 RFP1 cultures grown on 30 g/L cellulose in (A) 250 mL flasks or (B) a BioLector® a 48 RoundWellPlate.** Microscope pictures show calcofluor stained mycelium, the inset shows a macro photograph of a 1/16 dilution of the respective culture broth in a BioLector® well. Both cultures grew as homogeneous dispersed mycelium with a mixture of thin and thick hyphae at microscopic level.

**Application Example A: Estimation of cellulase induction by online fluorescence measurement using a CBH1 promoter-based RFP reporter construct:** To demonstrate the usefulness of the online fluorescence monitoring capabilities of the BioLector® in the context of cellulase production screening, the used *T. reesei* RUT-C30 strain was transformed with a reporter construct driving RFP expression under the control of a shortened version of the CBH1 cellulase promotor. The shortened version of the promotor results in weak constitutive RFP expression, that is increased under cellulase inducing conditions [12]. Figure 3 shows the corresponding online profiles of the RFP measurements for the BioLector® cultivations shown in figure 1. The RFP signal increased with increasing induction capability of the used carbon source and hence correlated with the cellulase production as evaluated by the filter paper assay. Such reporter strains of *T. reesei* can therefore be suitable to screen other carbon sources for their cellulase induction capability at high throughput in the BioLector®.

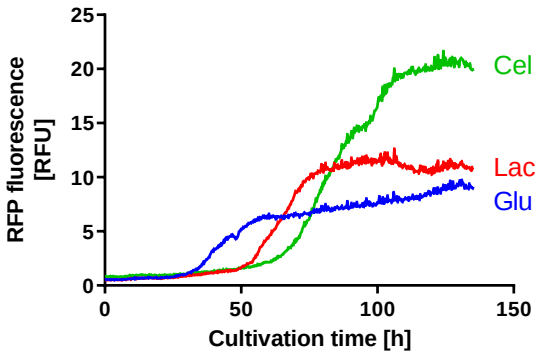



**Figure 3:RFP fluorescence profiles of *T. reesei* RUT-C30 RFP1 during cultivation on different carbon sources.** Fluorescence data show mean values of duplicate wells.

**Application Example B: Investigation of growth rates at different pH conditions**

The scattered light signal of the BioLector® can be used as an online approximation for biomass growth (see figure 4), which in turn can be used to calculate growth rates. The growth rate of *T. reesei* was compared at two different pH values. Thereby, it was shown that *T. reesei* achieves a higher $\mu_{max}$ at pH 4,8 than at pH 7 as shown in figure 5. The determined values are in agreement with growth rate trends determined by other authors.[13]

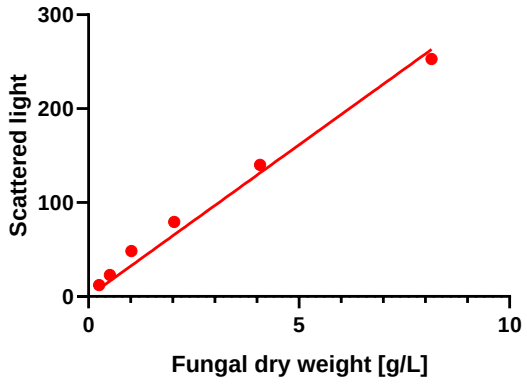



**Figure 4: Calibration of cell dry weight and scattered light for *T. reesei* RUT-C30 RFP1 grown on PIPPS buffered medium containing 30 g/L cellulose.** The culture was harvested after 120h of cultivation, when cellulose was consumed. Dry weight values for the different dilutions are derived from a triplicate measurement of the undiluted culture. Scattered light values show means of three sequential measurements.

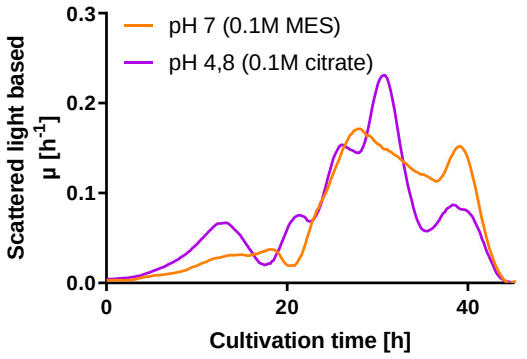



**Figure 5: Comparison of growth rates of *T. reesei* RUT-C30 RFP1 at pH7 (0,1 M MES buffer) and pH 4,8 (0,1 M citrate buffer).** *T. reesei* was grown on mineral medium containing 30 g/L glucose as carbon source and 0.1 M of the indicated buffer substances. Data show mean values of triplicate wells, dotted lines show standard deviation.

**Application Example C: Medium optimization for increased cellulase production:** To validate the throughput and reproducibility that can be achieved using the BioLector® system, cellulase production was investigated in relation to 16 different media variations in biological triplicate on one 48-well plate. The influence of phosphate, inorganic nitrogen as well as peptone as complex nitrogen source was investigated. The resulting cellulase production as quantified by the filter paper assay is shown in figure 6. It was found cellulase production was proportional to the used $(NH_4)_2SO_4$ while phosphate concentration had little effect on cellulase production. Addition of peptone clearly boosted cellulase production compared to defined media without peptone.

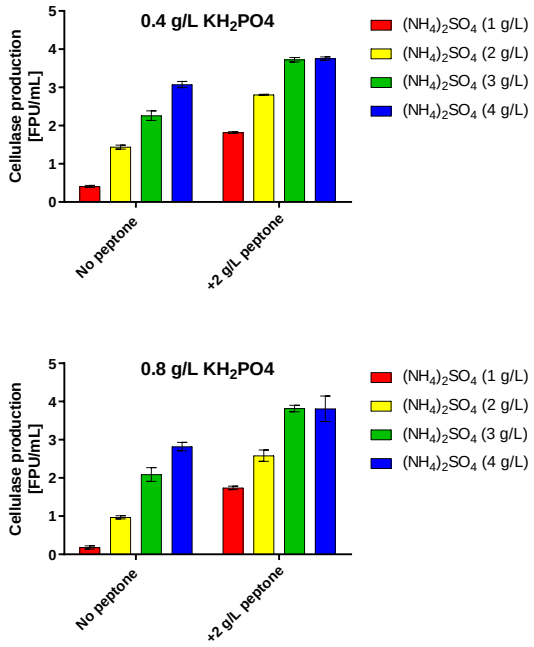



**Figure 6: Influence of different media compositions on cellulase production of *T. reesei* RUT-C30 RFP1.** Data show mean values of biological triplicates, error bars show standard deviation.

## Conclusion

This application note demonstrates various application examples for fungal cultivation processes in the BioLector®. We show that the BioLector® is a suitable screening device for filamentous fungi and we can validate the comparability between BioLector® and shake flask cultivations. The BioLector® is a reliable cultivation device for online reporter protein screening in high-throughput using non-invasive fluorescence measurements. We also validate the high-throughput and reproducibility by media optimization for increased cellulase production in *T. reesei.*

## Acknowledgments

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